

Molecular Epidemiology of Serogroup A Meningitis in Moscow, 1969–1997

Mark Achtman,* Arie van der Ende,† Peixuan Zhu,* Irina S. Koroleva,‡
Barica Kusecek,* Giovanna Morelli,* Ilse G.A. Schuurman,† Norbert Brieske,*
Kerstin Zurth,* Natalya N. Kostyukova,§ and Alexander E. Platonov‡

*Max-Planck Institut für Molekulare Genetik, Berlin, Germany; †Academic Medical Center, Amsterdam, the Netherlands; ‡Central Research Institute of Epidemiology, Moscow, Russia; and §N.F. Gamaleya Institute of Epidemiology and Microbiology, Moscow, Russia

Molecular analysis of 103 serogroup A *Neisseria meningitidis* strains isolated in Moscow from 1969 to 1997 showed that four independent clonal groupings were responsible for successive waves of meningococcal disease. An epidemic from 1969 to the mid-1970s was caused by genocloud 2 of subgroup III, possibly imported from China. Subsequent endemic disease through the early 1990s was caused by subgroup X and then by subgroup VI, which has also caused endemic disease elsewhere in eastern Europe. A 1996 epidemic was part of the pandemic spread from Asia of genocloud 8 of subgroup III. Recent genocloud 8 epidemic disease in Moscow may represent an early warning for spread of these bacteria to other countries in Europe.

Until the mid-1970s, *Neisseria meningitidis* expressing the serogroup A capsular polysaccharide caused numerous epidemics of meningitis and septicemia, with annual incidence rates >100/100,000 (1). During serogroup A epidemics, these bacteria can also be isolated from the nasopharynx of a considerable proportion of the healthy population (2). After the mid-1970s, large serogroup A epidemics became rare, except in the so-called Meningitis Belt in the Sahel region of Africa (3,4). In 1997, *N. meningitidis* caused approximately 500,000 cases of disease and 50,000 deaths worldwide, half of them in Africa (5). Serogroup A meningococci are currently isolated only rarely in western Europe and the United States, and a rise in either the healthy carriage rate or the proportion of serogroup A cases would be cause for concern.

Epidemic isolates of *N. meningitidis* have been assigned to a limited number of clonal groupings, historically by multilocus enzyme electrophoresis (MLEE) (4) and currently by multilocus sequence typing (MLST) (6). For MLST, sequences of housekeeping gene fragments are assigned different numeric allele designations, even if they differ by only one nucleotide. The combination of alleles from seven housekeeping gene fragments is called the sequence type (ST). Serogroup A bacteria have also been assigned to clonal groupings by random amplification of polymorphic DNA (RAPD) (7). These three methods yield largely concordant results, except that MLST is the most conservative and RAPD differentiates the largest number of isolates. The clonal groupings among serogroup A meningococci have been designated subgroups I through IX according to MLEE (7,8). Some of these subgroups have not been differentiated by RAPD analysis (I vs. II, III vs. VIII) (7) or MLST.

Recent epidemic serogroup A disease in Europe has largely reflected the pandemic patterns of spread of subgroup III meningococci. After a large subgroup III epidemic in China in the mid-1960s (8), these bacteria caused an outbreak of meningococcal disease in 1969 in western Norway (9), followed in the mid-1970s by a major epidemic in Finland (10). During the 1970s, subgroup III meningococci were isolated throughout western and northern Europe (11). In 1987, serogroup A, subgroup III meningococci caused a meningitis epidemic during the Hajj pilgrimage to Mecca, Saudi Arabia (12), and healthy pilgrims carried these bacteria throughout the world. Localized serogroup A disease was associated with former pilgrims for several years in the United Kingdom (13) and France (14), but these meningococci have since largely disappeared from western Europe. Rare endemic serogroup A disease was associated with subgroup VI in East Germany in the 1980s (8) and with subgroup IX in the Netherlands in the early 1990s (7).

Meningococci are naturally transformable and can import novel alleles from *neisseriae* that colonize the nasopharynx (15,16). However, these recombinant strains are usually lost during the bottlenecks caused by limited numbers of bacteria being transmitted during epidemic spread (17). Occasionally, variants pass through these bottlenecks first, and all subsequent progeny have a common genetic background different from that of the parental bacteria (17). Most subgroup III bacteria isolated from the mid-1960s to the mid-1980s ("pre-Mecca") had particular alleles of the hypervariable *opaB* and *opaD* loci encoding Opa (opacity) proteins and the *iga* locus encoding IgA1 protease. In contrast, subgroup III bacteria isolated after the 1987 Mecca epidemic ("post-Mecca") had other alleles at these three loci (17). Exceptional isolates with still other alleles at one or more of these loci were also found in several countries, but these variants all disappeared during subsequent epidemic spread (17).

Address for correspondence: Mark Achtman, Max-Planck Institut für Infektionsbiologie, Schumannstr. 21/22, 10117 Berlin, Germany; fax: +49 30 28460 750; e-mail: achtman@mpiib-berlin.mpg.de

Data in the English literature about meningococci from Russia and other areas of eastern Europe are scarce. In recent decades, most patients with meningitis in Moscow (population 8.6 million during 1990 to 1999) have been treated at the Second Moscow Hospital for Infectious Diseases. Our review of their records shows that from 1980 to 1999, the annual incidence rates of meningitis caused by *Haemophilus influenzae* and *Streptococcus pneumoniae* were approximately 0.2/100,000 and 0.4/100,000, respectively. In contrast, several epidemics of meningococcal disease with much higher incidence rates occurred in Moscow from 1924 to 1998 (Figure 1). Large serogroup A epidemics occurred in 1931 and 1940, followed by a decline in disease incidence until

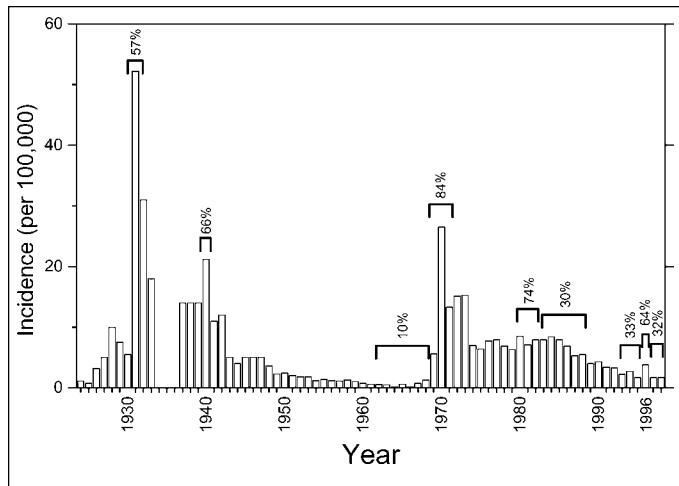


Figure 1. Annual incidence rates of meningococcal meningitis in Moscow from 1924 to 1998. Incidence rates were extracted from the compilation by Bolshakov (18) and data published in annual reports of the Russian Ministry of Public Health or obtained from 1969 to 1998 by the Central Research Institute of Epidemiology from epidemiologic investigations in Moscow. The percentages of serogroup A meningococci among all meningococci isolated from disease were compiled from various sources [number of strains]: 1924-1969, Bolshakov (18); 1962-1971, Kostyukova (19) [300]; 1980-1988, Demina (20) [1,000]; and 1993-1998, Koroleva, laboratory records [500].

the late 1960s. In 1968, a serogroup A epidemic began in Lipezk, Russia, where factory workers had recently arrived from Vietnam. These immigrants had traveled by rail through China, where subgroup III disease was prevalent (8). The incidence rate of meningococcal disease in Moscow and the proportion of serogroup A isolates remained high until the 1980s. In 1996, a new outbreak of serogroup A disease began, again initially associated with the Vietnamese community. During the outbreak peak in early 1996, 50 (34%) of 147 patients with meningococcal disease were Vietnamese, and all their disease isolates were serogroup A. During the rest of 1996, only 8 (6%) of 145 patients with meningococcal disease belonged to the Vietnamese community. During 1997 to 1999, hundreds of thousands of Muscovites were immunized with A polysaccharide vaccine. The incidence rate and the proportion of serogroup A isolates decreased and have remained stable through 1999.

We present data on the molecular epidemiology and genetic relationships of serogroup A meningococci isolated in Moscow from 1969 to 1997. The data show that four clonal

groupings have been responsible for successive waves of disease and that epidemic disease in Moscow since 1994 is associated with a new subgroup III pandemic.

Materials and Methods

Serogroup A Isolates from Moscow

One hundred three strains of *N. meningitidis* were isolated in Moscow from the cerebrospinal fluid or blood of patients with systemic meningococcal disease or from throat swabs of their close contacts (seven subgroup VI strains, 1988-89). Strains were serogrouped by immune precipitation with specific sera and lyophilized. The bacteria we describe represent almost all the viable serogroup A bacteria still available in the laboratory collections at the Central Research Institute of Epidemiology and the Gamaleya Institute of Epidemiology and Microbiology, with the following exceptions: Only one third of the bacteria available from 1969 to 1972 and half the bacteria from 1996 were tested to avoid bias toward these two periods. The lyophilized bacteria were purified again by single colony isolation. These bacterial cultures were then serogrouped, serotyped, and subtyped by using whole-cell enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies (8). DNA was prepared as described (21) and used for molecular fine typing and MLST.

MLST

Both strands of fragments of *abcZ*, *adk*, *aroE*, *gdh*, *pdhC*, *pgm*, and *fumC* were sequenced as described (<http://www.mlst.net>), except that primers O1101 (5'TCCGGCT-TGCCGTTTGTCTAG) and O1102 (5'TTGTAGGCGGTTTTG-GCGAC) were used for *fumC*. At the beginning of this project, the MLST database inadequately represented the diversity of serogroup A meningococci because it contained data for only 36 strains, predominantly from MLEE subgroups I, III, and IV-1 (6). To enlarge the database, MLST was performed with 66 serogroup A isolates from diverse sources that had previously been assigned to subgroups II through IX by MLEE (7,8) and in some cases by RAPD analysis (7). MLST was also performed with 42 serogroup A isolates from Moscow chosen from the most diverse branches of the RAPD tree. Other laboratories had deposited data for eight serogroup A strains in six novel STs, resulting in 152 serogroup A strains in 31 STs (Table 1). The results, including strain description, source, and MLEE assignments, are publicly available (<http://www.mlst.net>).

Molecular Fine Typing of Subgroup III

Alleles of the four chromosomal *opa* genes were determined by restriction analysis of polymerase chain reaction (PCR) products generated by using one primer in the upstream conserved flanking DNA and a second HV2-specific primer, as described (17). The complete *opa* genes were also sequenced from selected representative strains.

A fragment of *iga* was amplified from chromosomal DNA by using primers O103 and O115 and tested for a *Bfa*I site that is present in *iga1* but not in *iga2* or *iga3* (17). A different fragment of *iga* was amplified with primers O113 and O151 and tested for a *Dde*I site that is present in *iga3* but not *iga2* or *iga1* (17). In addition, the entire *iga* gene was amplified with primers O103 and O152 and sequenced from one ST5 and one ST7 strain.

Table 1. Multilocus sequence typing analysis of serogroup A meningococci

ST ^a	MLEE/ RAPD	Sources	No.	<i>fumC</i>	<i>abcZ</i>	<i>adk</i>	<i>aroE</i>	<i>gdh</i>	<i>pdhC</i>	<i>pgm</i>
21	other	UK (1941)	1	1	1	5	1	2	16	17
1	I, II	Pandemic (1963-80)	15	1	1	3	1	1	1	3
75	X	Moscow (1983-94)	3	1	2	3	25	1	1	3
78	X	Moscow (1997)	1	1	2	3	25	1	1	17
76	X	Moscow (1983-85)	4	17	2	3	1	1	1	3
77	X	Moscow (1984-85)	3	1	2	3	1	1	1	3
69	VI	Moscow (1970)	1	28	1	3	4	1	1	3
2	VI	Moscow, GDR (1980-95)	17	7	1	3	4	1	1	3
68	VI	Moscow (1994-96)	2	7	1	3	4	21	1	17
70	VI	Moscow (1984-86)	2	7	1	3	4	22	21	3
73	VI	Moscow (1995)	1	7	1	3	4	1	21	3
58	VI	GDR (1986)	1	7	1	3	21	1	1	3
71	VI	Moscow (1985)	1	7	1	3	10	1	1	3
72	VI	Moscow (1987)	1	7	1	3	7	1	1	3
57	II	USA (1930-43)	2	1	1	3	1	1	2	3
4	IV-1,IV-2	Pandemic (1917-90)	37	1	1	3	3	4	2	3
80		Gambia (1983)	1	31	1	3	15	4	2	3
203		Gambia (1996)	1	1	1	3	2	3	2	3
5	III,VIII	Pandemic (1963-98)	24	1	1	1	2	3	2	3
6	III	China (1966)	1	1	1	1	2	3	2	11
7	III	Pandemic (1992-96)	9	1	1	1	2	3	2	19
3	V,VII	China (1963-87)	11	1	1	3	1	1	23	13
59	V	China (1980)	1	22	1	14	1	1	23	13
103		Greece (1996)	1	17	8	4	6	5	18	2
60	IX	Holland (1989-92)	4	17	17	5	19	3	26	2
61	IX	Holland (1992)	1	17	17	10	19	3	26	2
79	other	Moscow (1995)	1	26	12	5	6	9	22	8
299		Czechoslovakia (1972)	1	26	2	29	36	26	18	20
300		Czechoslovakia (1972)	1	26	2	29	9	26	18	20
388		Czechoslovakia (1972)	2	35	57	2	2	6	68	12
400		Czechoslovakia (1972)	1	43	12	16	2	3	37	7

^aST = sequence type; GDR = German Democratic Republic (East Germany); MLEE = multilocus enzyme electrophoresis; RAPD = random amplification of polymorphic DNA.

The IS1106A locus near *opcA* was amplified by using primers in conserved DNA flanking this locus (22). Two sizes of PCR products (4.8 and 3.5 kb) were obtained among the subgroup III strains. (One strain yielded a 12-kb product that was not investigated further.) An internal, polymorphic 307-bp stretch was sequenced from one product of each size and found to differ by 19 bp, including four restriction sites for *Hae*III, *Cla*I, and *Bcl*I. The allele in the 4.8-kb fragment was named IS1106A10, and the allele in the 3.5-kb fragment was named IS1106A7. PCR products from all the subgroup III strains were tested for the four distinctive restriction sites, but no additional diversity was identified. Details on alleles at this locus in subgroup III bacteria from sources other than Moscow have been described (23).

The 2.1-kb *tbpB* gene was amplified by using an internal primer located near the ATG start codon (3'-Met2) and a primer located in the intergenic region between *tbpB* and *tbpA* (5'-Inter1), as described (16). Different alleles of *tbpB* were recognized by digesting the 2.1-kb fragment with *Hae*III, *Msp*I, and *Ssp*I and by sequencing the entire fragment from two ST5 strains and one ST7 strain. The *tbpB* allele designations are congruent with those described for a 600-bp 5'-terminal region that has been examined from numerous other strains (16).

To distinguish between *pgm3* and *pgm19* (6), the *pgm* locus was PCR-amplified by using primers O781 (5'-CGGCGATGCCGACCGCTTGG) and O782 (5'-GGTGATGATTTTCGGTTGCGCC). The PCR products were tested for an *Hinf*I site that is absent in *pgm3* and present in *pgm19*.

Results

Genetic Relationships of Serogroup A Strains from Moscow

One hundred three serogroup A *N. meningitidis* strains isolated in Moscow during 1969 to 1998 were investigated by RAPD analysis to elucidate their genetic relationships. The data (Figure 2) revealed three primary clusters of isolates, two of which (subgroups III and VI) were assigned to known clonal groupings by comparison with previous data (7). The third cluster (24 isolates) differs from previously defined subgroups and was therefore tentatively designated subgroup X. Two singleton strains were labeled IV (a strain formerly assigned to subgroup IV-2 by MLEE) and ST79.

Forty-two strains that represent the diversity revealed by RAPD were analyzed by MLST. These data were combined with data from other strains as described in Materials and Methods, resulting in a database containing 152 serogroup A isolates (Table 1, Figure 3). MLST did not distinguish several subgroups defined by MLEE (I and II, IV-1 and IV-2, III and VIII, V and VII). Otherwise, the subgroup structure in a neighbor-joining (NJ) tree of the fraction of alleles differing between STs (Figure 3) was largely concordant with prior subgroup assignments from MLEE (8) or RAPD (7). Only STs 1 and 57 clustered inappropriately in the NJ tree (Figure 3).

The MLST results confirmed the assignments of isolates from Moscow to subgroups III and VI. They also demonstrated that subgroup X is distinct from previously defined subgroups and is closely related to subgroups I and II. Individual STs of

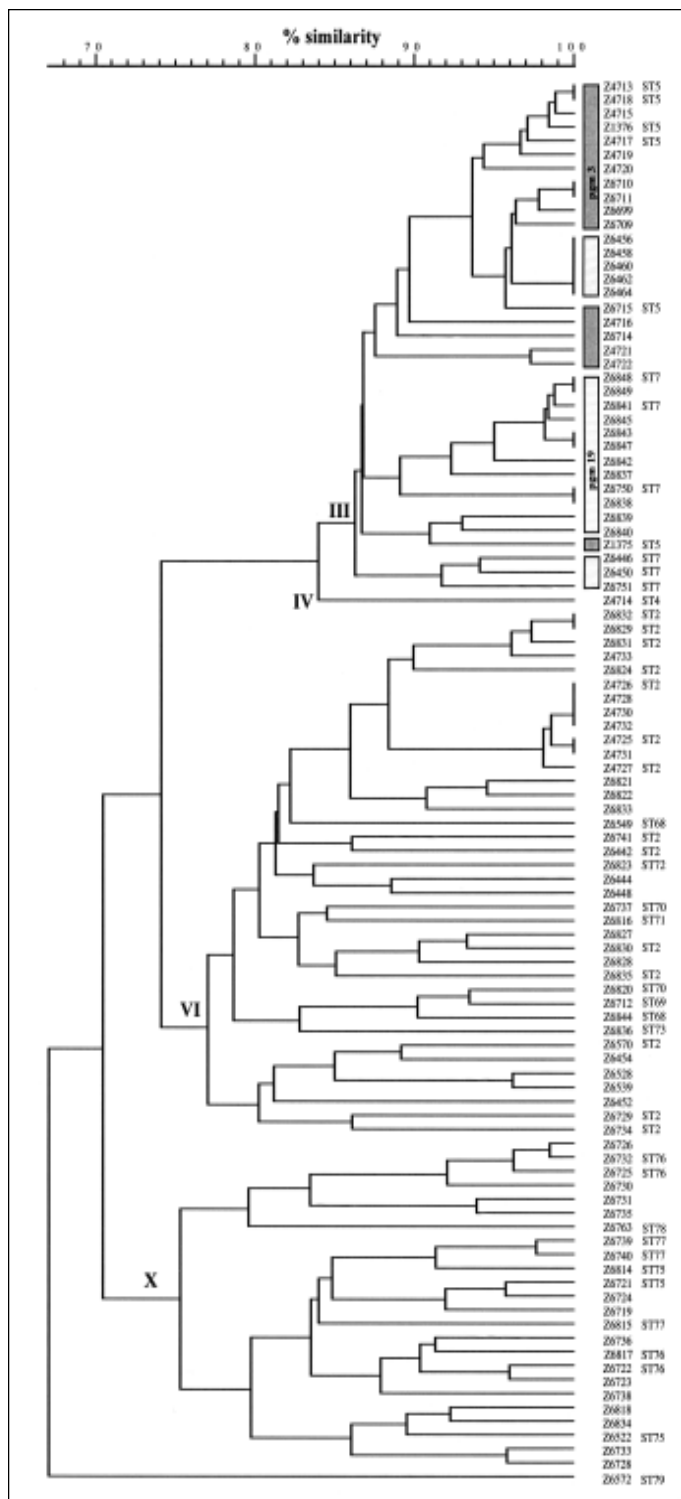


Figure 2. UPGMA tree of band differences between random amplified polymorphic DNA (RAPD) patterns from 103 serogroup A strains from Moscow. RAPD tests were performed with primers 1254, 1281, NM03, and NM04 as described (7), generating four patterns for each strain. The combination of the differences between these patterns was used to generate a UPGMA tree with the program GelCompar (Applied Maths, Kortrijk, Belgium), as described (7). Subgroup designations are indicated within the tree. The strain designations and the sequence type (ST) assignments from MLST analysis are shown at the right. For subgroup III bacteria, dark gray rectangles indicate *pgm3* and light gray rectangles indicate *pgm19*. Strain Z4714 is indicated as belonging to subgroup IV because RAPD does not distinguish between subgroups IV-1 and IV-2.

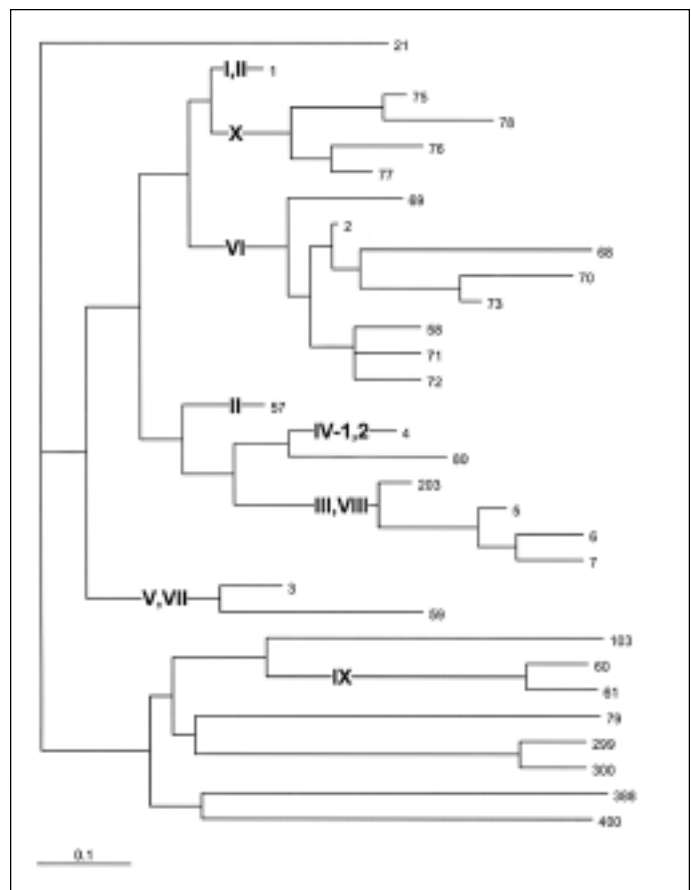


Figure 3. Midpoint rooted neighbor-joining (NJ) tree of the proportion of seven housekeeping gene fragments that differed between individual sequence types (STs) among 152 serogroup A isolates. The ST designations are indicated at the right of each twig, and the subgroup designations are shown in bold print in the tree. A scale bar showing the distance of 0.1 is at the lower left. STs 1 and 57, containing six subgroup II strains, are widely separated in this tree although they differ only at the *pdhC* locus (Table 1). These two STs were on adjacent twigs in a UPGMA tree (data not shown), but that tree separated STs 3 and 59 (subgroup V), that differ only at two of the seven loci (Table 1) and are on neighboring twigs in the NJ tree. Given the low number of allelic differences between STs 1 and 57 and STs 3 and 59, their aberrant relative positions in one or the other tree were ignored and their former subgroup assignments have been retained.

these three subgroups share identical alleles for up to six of the seven gene fragments sequenced (Table 1).

Inconsistent Fine Typing by RAPD and MLST

Subgroup III isolates from Moscow were isolated during more than one epidemic wave, raising the question whether these isolates shared a common source. However, the concordance between RAPD and MLST did not extend below the subgroup level. The two major RAPD branches in subgroup III initially seemed to correspond to MLST ST5 and ST7. These STs are identical except for their *pgm* alleles (ST5, *pgm3*; ST7, *pgm19*), which differ by 19 (4%) of 450 bp, including a distinctive restriction site. Genotyping of *pgm* from all subgroup III isolates by PCR-restriction fragment length polymorphism (PCR-RFLP) showed that both major RAPD branches contained both *pgm* alleles (Figure 2). Therefore, additional powerful fine typing methods, such as

multilocus PCR-RFLP, were needed to resolve whether all bacteria within a subgroup originated from the same sources.

Serologic Properties

In some cases, serologic serotyping and serosubtyping can be used to enable rapid recognition of clonal groupings. The serologic properties of all the serogroup A bacteria from Moscow were determined by ELISA with monoclonal typing antibodies. The results confirmed previous results for subgroups III and VI (8) and showed that subgroup X is 4,21:P1.10 or 21:P1.10 (Table 2). Subgroup X is therefore not distinguishable by serologic testing from serogroup A subgroups I, II, IV-2 and VI, which are also 4,21:P1.10. The subgroup III strains uniformly expressed the class I and IIa pilin epitopes (8); however, the pilin epitopes of subgroups VI and X were not uniform: half of each group of strains expressed class I and IIa epitopes and the other half the class IIb epitope. Thus, except for subgroup III, serologic typing would not be useful for distinguishing these various subgroups.

Table 2. Serologic properties of serogroup A meningococci from Moscow

Subgroup	Proportion (%)	Serotype	Serosubtype
III	34/38 (89)	21	P1.20,9
VI	6/39 (15)	21	P1.10
VI	12/39 (31)	4,21	P1.10
VI	17/39 (44)	4,21	P1.5,10
X	17/24 (71)	4,21	P1.10
X	6/24 (25)	21	P1.10

Successive Waves Caused by Different Clonal Groupings

The three subgroups were isolated in Moscow during four successive waves of disease caused, respectively, by ST5 of subgroup III (1969 to 1977, 17 of 19 isolates), subgroup X (1983 to 1985; 21 of 27), subgroup VI (1986 to 1995; 32 of 36) and ST7 of subgroup III (1996-97; 19 of 21) (Table 3). The isolation of subgroup III bacteria correlated with epidemic peaks of disease in 1970 and 1996 (Figure 1); in both cases, subgroup III bacteria were also isolated in Moscow 1 to 2 years before the peak epidemic year (Table 3). In contrast, the isolation of subgroup X or subgroup VI did not correlate with an increase in the incidence rate for serogroup A disease. The slight increase in disease incidence in the mid-1980s was associated with serogroup B meningococci.

Molecular Fine Typing of Subgroup III Isolates

Both pre-Mecca and post-Mecca isolates are ST5, while the subgroup III meningococci isolated in Moscow since the mid-1990s are ST7. PCR-RFLP of the *opaB*, *opaD* and *iga* loci distinguished pre-Mecca from post-Mecca bacteria (17) but did not allow unambiguous assignment of the isolates from Moscow to either of these two waves. To resolve the fine structure of subgroup III, we identified three additional distinctive molecular markers, *tbpB*, IS1106A, and *pgm*, which were tested by PCR-RFLP plus sequencing for numerous subgroup III isolates from other countries (23). PCR-RFLP of these six variable loci subdivides subgroup III bacteria into nine so-called genoclouds, each containing a spectrum of related genotypes, and shows that a third wave of pandemic disease caused by genocloud 8 began in eastern

Table 3. Years of isolation of individual subgroups of serogroup A *Neisseria meningitidis* in Moscow

Year	Number of strains					Total
	III (ST5 ^a)	X	VI	III (ST7)	other	
1969	1					1
1970	9		1			10
1971	4				1	5
1973	1					1
1977	2					2
1983		4				4
1984		14	3			17
1985		3	3			6
1986			3			3
1987			3			3
1988			8			8
1991			8			8
1993			2			2
1994		1	4	1		6
1995			4	1	1	6
1996			1	17		18
1997		1		2		3
Total	17	23	40	21	2	103

^aST = sequence type

Subgroup assignments are according to data from random amplification of polymorphic DNA (Figure 2). For strains of subgroup III where a complete multilocus sequence typing analysis was not performed, bacteria were assigned to ST5 and ST7 on the basis of results with *pgm* polymerase chain reaction/restriction analysis. Other: 1971, subgroup IV-2; 1995, ST79.

Asia in the early 1990s. Genocloud 8 bacteria differ from other subgroup III meningococci in having the *tbpB55*, *pgm19*, and IS1106A7 alleles (Table 4). All 21 subgroup III bacteria isolated in Moscow from 1994 to 1997 also have these alleles (Table 4) and thus represent an extension of the third pandemic wave.

The subgroup III ST5 meningococci isolated in Moscow from 1969 to 1977 are distinct from all other subgroup III meningococci and define genocloud 2 (Table 4). The most closely related genocloud is genocloud 1, isolated in China in the mid-1960s, which contains the same IS1106A10 element but differs at *tbpB* and *opaB*. The genocloud 3 bacteria that caused outbreaks in northern Europe concurrently with the epidemic in Moscow differed in IS1106A (IS1106A4) as well as *tbpB* and *opaB*. Other subgroup III genoclouds, including genocloud 5 (post-Mecca), differed at even more loci (Table 4).

Phylogenetic Ancestry of Genocloud 2

The unique *tbpB* allele in ST5 subgroup III bacteria from Moscow is not informative for phylogenetic descent: It was not found in any other genocloud and was probably imported by DNA transformation (16) shortly before or after these bacteria arrived in Moscow. However, the phylogenetic history of the unique *opaB* alleles in the subgroup III bacteria from Moscow can be reconstructed from their sequences. Genocloud 1 bacteria from China have the *opa132*, *opa92*, *opa131*, and *opa101* alleles at the *opaA*, *B*, *D*, and *J* loci, respectively. The novel alleles at the *opaB* and *opaD* loci in genocloud 2 bacteria from Moscow probably represent gene conversion with the *opaJ101* and *opaA132* alleles (Figure 4). The *opaB140* allele, which was present in all but one strain from Moscow (Table 4), differs from *opaJ101* by only one nucleotide (Figure 4). The *opaB113* allele (one strain) can be explained by gene conversion of *opaB140* with DNA from the *opaA132* allele. *opaD131* is present in genocloud 1 and was

Table 4. Hypervariable alleles among subgroup III meningococci isolated in Moscow

Strains	No.	Locus							
		<i>tbpB</i>	<i>iga</i>	<i>pgm</i>	<i>IS1106</i>	<i>opaA</i>	<i>opaB</i>	<i>opaD</i>	<i>opaJ</i>
^a genocloud 1		1	2	3	A10	132	92	131	101
genocloud 3		1	2	3	A4	132	92	131	101
genocloud 5		1	3	3	A4	132	94	100	101
genocloud 8		55	2	19	A7	132	92	131	101
Moscow genocloud 2									
1969-1973	14	38	2	3	A10	132	140	131	101
1970	1	38	2	3	A10	132	113	131	101
1977	1	38	2	3	A10	132	140	110	101
1977	1	38	2	3	nd ^b	132	140	109	101
Moscow genocloud 8									
1994-1997	21	55	2	19	A7	132	92	131	101

^aData for the genoclouds at the top of the table are from a separate manuscript (23). Sources of genoclouds: 1, China, 1966; 3, pre-Mecca isolates (1969-1984) from Europe, Brazil and China; 5, post-Mecca isolates (1987-1998) from Africa and pilgrims returning from Mecca in 1987 to various countries; and 8, China, Mongolia, and Africa, 1993-2000. All serogroup A isolates from Moscow that were assigned to subgroup III by random amplification of polymorphic DNA are included in this table. The *opaA* and *opaJ* alleles have not been tested in all bacteria from the various genoclouds because they are otherwise so uniform.

^bnd = not determined because this strain yields an unusual 12-kb polymerase chain reaction product for the *IS1106A* region.

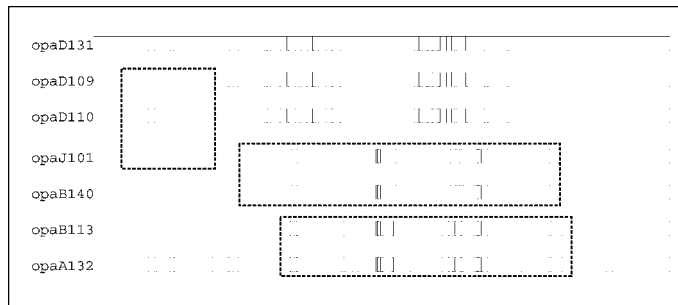


Figure 4. Sequence differences among *opa* alleles found in genocloud 2 subgroup III serogroup A meningococci from Moscow. Each vertical line represents a nucleotide different from the consensus sequence. Hatched rectangles indicate identical stretches that are inferred to represent gene conversion.

also found in all but two genocloud 2 strains from Moscow. The two exceptional strains contain the *opaD109* and *opaD110* alleles, which could easily have arisen by gene conversion of part of the *opaD131* locus with DNA from the *opaJ101* locus.

Discussion

Since the 1920s, serogroup A meningococci have been responsible for a large proportion of the bacterial meningitis in Moscow (Figure 1). Serogroup A meningococci isolated in Moscow from 1969 to 1997 (103 isolates) were analyzed by RAPD. Strains representing the diversity revealed by the RAPD data were analyzed by MLST (44 isolates), and all 38 subgroup III strains were subjected to molecular fine typing by multilocus PCR-RFLP. The results illustrate the limitations of these methods and yield important information about the phylogenetic relationships among serogroup A meningococci. They also demonstrate that serogroup A meningococcal disease in Moscow since 1969 has been caused by four successive, independent waves of bacterial colonization, most recently by the spread of a third subgroup III pandemic wave from East Asia.

Typing Methods

RAPD analysis was used to provide a quick overview of the genetic diversity in the 103 strains investigated (Figure 2). This information was used to select a smaller group of bacteria to be tested by more expensive and labor-intensive methods such as MLST. As in previous comparisons

between RAPD and MLEE (7), the subgroup assignments were concordant between MLST and RAPD. The RAPD data allowed the initial recognition of a new subgroup, subgroup X, and the MLST data confirmed that subgroup IX is not genetically related to the epidemic serogroup A subgroups as originally demonstrated by RAPD data (7). Thus, RAPD analysis can be a highly reliable, quick method for recognizing genetically related bacterial groups.

Finer subdivisions in subgroups III and X, suggested by the RAPD data, were contradicted by MLST results. ST76 was distributed among both primary RAPD subdivisions of the subgroup X cluster, and the two primary RAPD subdivisions of the subgroup III cluster did not correlate with presence of *pgm3* versus *pgm19* (Figure 2) or with epidemiologic data. Such random noise will probably be most extreme for bacteria such as *N. meningitidis*, which frequently import DNA from unrelated neisseriae (15). Thus, putative subdivisions within phylogenetically related groups suggested by RAPD data should be confirmed by independent methods.

MLST is based on sequences of conserved housekeeping genes and sequence variants are rarer than with most other methods used in molecular epidemiology, thus facilitating global, long-term epidemiology (6). For subgroup III, the distinction between STs 5 and 7 based on *pgm* is a very useful molecular marker for recognizing bacteria from the third pandemic wave. However, the epidemiologic data do not suggest that other STs are useful markers for genetic variants that have spread extensively, and MLST does not necessarily provide the discrimination desired for fine typing.

Unfortunately, the fine structure of bacteria that frequently import foreign DNA is very difficult to resolve, and we know of no method that is eminently suitable for this task. Our data argue against the use of RAPD and MLST. Although pulsed-field gel electrophoresis is highly suitable for differentiating almost identical bacteria in limited numbers of strains (17,24,25), it is poorly suited for elucidating the relationships among large numbers of less strongly related bacteria. Fine typing was therefore attempted based on multilocus RFLP of PCR fragments of *pgm* plus the variable genes *opa*, *iga*, and *tbpB* and the *IS1106A* element. Multilocus PCR-RFLP of variable genes allowed molecular fine typing within subgroup III meningococci, but the data are also limited by considerable random noise, resulting in genoclouds of related variants (23). Alternative strategies involving IS

elements (26), amplified fragment-length polymorphism (27), or variable nucleotide repeat sequences (28) have been used successfully for fine typing of highly uniform microorganisms. Such methods have not yet been extensively tested with *N. meningitidis* but seem likely to be limited by at least as much random noise as the methods we used. Thus, reliable fine typing remains difficult to achieve.

Phylogeny of Serogroup A Meningococci

The MLST data on 152 serogroup A strains allow inferences about the evolution of these bacteria. The STs in subgroups I through VIII and X (the epidemic subgroups) probably stem from a common ancestor. They form a monophyletic clade in phylogenetic trees (Figure 3), and many of these STs share the *fumC1*, *abcZ1*, *adk3*, *gdh1*, *pdhC1*, and *pgm3* alleles (Table 1). The epidemic subgroups include meningococci isolated since the beginning of this century (ST4, subgroup IV-2, 1917; ST57, subgroup II, 1930) (8) and must have evolved before then.

Older isolates of subgroups III (1960s), IV-1 (1960s), and IV-2 (1917) had identical or nearly identical *opa*, *iga*, and *tbpB* alleles (16,17), whereas more recent isolates have imported novel alleles at these loci. These data indicate that the common ancestor of these three subgroups existed approximately 100 years ago (17).

The branch lengths (Figure 3) and common alleles indicate that the epidemic subgroups are probably not much older than the split between subgroups III, IV-1, and IV-2. The epidemic serogroup A subgroups may therefore have descended from a common ancestor that existed within the last few hundred years. Epidemic meningitis was first observed in Geneva in 1805 (29), and that epidemic may have marked the evolution of serogroup A after the import of the *sacA-D* genes encoding the A polysaccharide. Horizontal exchange between serogroups B and C of genes encoding capsular polysaccharides has been documented (30), and more than one capsular polysaccharide serogroup is present in several of the otherwise uniform STs in the complete MLST database. Furthermore, the *sacA-D* genes are characterized by an unusually low GC content (31), as if these genes were recently imported from an unrelated species. If this speculation is correct, the other serogroup A bacteria (Figure 3, bottom) may represent unrelated *N. meningitidis* that have imported *sacA-D* genes from the epidemic subgroups. Alternatively, the *sacA-D* genes may have been imported from an unrelated species more than once, or the epidemic subgroups may have imported the *sacA-D* from one of the nonepidemic STs (Figure 3, bottom). Analysis of the sequence diversity of the *sacA-D* genes in various STs might resolve these alternative explanations.

Molecular Epidemiology

Evaluation of the long-term molecular epidemiology of bacterial pathogens within a global context is complex, requiring large, representative strain collections and reliable, preferably phylogenetically based, typing schemes. Our results illustrate both strengths and weaknesses in our knowledge of serogroup A epidemiology.

The assignment of genocloud 8 bacteria isolated in the mid-1990s in Moscow to the third subgroup III pandemic wave is straightforward and indisputable. It rests on three

unusual markers (*pgm19*, *tbpB55*, and IS1106A7), and no differences in these three markers were detected among recent subgroup III isolates from China, Mongolia, Africa, and Russia. The initial association of genocloud 8 disease in Moscow with the Vietnamese community remains unexplained.

The genocloud 2 subgroup III bacteria from the early 1970s differ at the *opaB* and *tbpB* loci from all known subgroup III meningococci. We argue that genocloud 2 is probably derived from genocloud 1 in China because of the shared IS1106A10 allele, which was not found in any other subgroup III bacteria. In support of this interpretation based on molecular data, this epidemic first broke out among Vietnamese immigrants who had traveled through China en route to Russia. However, few subgroup III strains exist that were isolated before 1969, and our knowledge about the diversity and patterns of serogroup A disease before 1969 is only fragmentary.

Subgroup VI bacteria of serogroups A, B, and C were occasionally isolated from endemic disease in East Germany in the 1980s (8). Our data show that most of the serogroup A meningococci isolated during 1986 to 1994 in Moscow were also subgroup VI, and these bacteria may have been widely distributed in eastern Europe in the 1980s. In contrast, subgroup X, common in Moscow during 1983 to 1985, has not been described previously. Various STs (Figure 3, bottom) have also been isolated only from single countries (subgroup IX in the Netherlands during 1989 to 1993 [7]; STs 299, 300, 388, and 400 in Czechoslovakia in 1972 [K. Jolley and P. Kriz, pers. comm.]; ST79 in Moscow in 1995; and ST103 in Greece in 1996 [R. Urwin, pers. comm.]). These bacteria have apparently not spread extensively, unlike the epidemic subgroups. In this respect, they resemble most endemic serogroup B meningococci, such as those identified by MLEE among endemic disease isolates from the Netherlands (32) and carrier isolates from Norway (33). Additional serogroup A STs specific to certain countries or regions probably remain to be identified. Current meningococcal strain collections containing a few thousand isolates are probably one or two orders of magnitude too small to yield reliable epidemiologic data for unusual STs that have not caused larger disease outbreaks.

Subgroup III has caused large disease outbreaks, and its molecular epidemiology has been extensively investigated (11,17,23). As a result, the available information on genetic changes during pandemic spread of these bacteria is among the most extensive for any bacterial pathogen. Our data show that the extensive pandemic spread of these bacteria is likely to continue. Although Moscow and western Europe differ somewhat in epidemiologic patterns, the recent extension of this third pandemic wave to Moscow warns us that we should be prepared for new, large serogroup A outbreaks in countries in both eastern and western Europe.

Acknowledgments

We gratefully acknowledge information on sources of strains in the MLST database and permission to cite these data from Keith Jolley and Rachel Urwin.

This work was partly supported by grants Ac 36/9-2 from the Deutsche Forschungsgemeinschaft and INTAS.

Dr. Achtman is a scientific group leader at the Max-Planck Institut für Infektionsbiologie, Berlin. His group investigates the population structures of bacterial pathogens, including *N. meningitidis*, *Helicobacter pylori*, and *Yersinia pestis*. These analyses integrate results from epidemiologic studies of global disease patterns.

References

- Peltola H. Meningococcal disease: still with us. *Rev Infect Dis* 1983;5:71-91.
- Crowe BA, Wall RA, Kusecek B, Neumann B, Olyhoek T, Abdillahi H, et al. Clonal and variable properties of *Neisseria meningitidis* isolated from cases and carriers during and after an epidemic in the Gambia, West Africa. *J Infect Dis* 1989;159:686-700.
- Achtman M. Molecular epidemiology of epidemic bacterial meningitis. *Rev Med Microbiol* 1990;1:29-38.
- Achtman M. Global epidemiology of meningococcal disease. In: Cartwright K, editor. *Meningococcal disease*. Chichester, England: John Wiley & Sons, Ltd; 1995:159-75.
- Tikhomirov E, Santamaria M, Esteves K. Meningococcal disease: public health burden and control. *World Health Statistical Quarterly* 1997;50:170-6.
- Maiden MCJ, Bygraves JA, Feil E, Morelli G, Russell JE, Urwin R, et al. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc Natl Acad Sci U S A* 1998;95:3140-5.
- Bart A, Schuurman IGA, Achtman M, Caugant DA, Dankert J, van der Ende A. Random amplified polymorphic DNA (RAPD) genotyping of serogroup A meningococci yields similar results to multilocus enzyme electrophoresis (MEE) and reveals new genotypes. *J Clin Microbiol* 1998;36:1746-9.
- Wang J-F, Caugant DA, Li X, Hu X, Poolman JT, Crowe BA, et al. Clonal and antigenic analysis of serogroup A *Neisseria meningitidis* with particular reference to epidemiological features of epidemic meningitis in China. *Infect Immun* 1992;60:5267-82.
- Hjetland R, Caugant DA, Hofstad T, Frøholm LO, Selander RK. Serogroup A *Neisseria meningitidis* of clone III-1 in western Norway, 1969-73. *Scand J Infect Dis* 1990;22:241-2.
- Mäkelä PH, Peltola H, Käyhty H, Jousimies H, Pettay O, Ruoslahti E, et al. Polysaccharide vaccines to group A *Neisseria meningitidis* and *Haemophilus influenzae* type b: a field trial in Finland. *J Infect Dis* 1977;136:43-50.
- Achtman M, Kusecek B, Morelli G, Eickmann K, Wang J, Crowe B, et al. A comparison of the variable antigens expressed by clone IV-1 and subgroup III of *Neisseria meningitidis* serogroup A. *J Infect Dis* 1992;165:53-68.
- Moore PS, Reeves MW, Schwartz B, Gellin BG, Broome CV. Intercontinental spread of an epidemic group A *Neisseria meningitidis* strain. *Lancet* 1989;ii:260-3.
- Jones DM, Sutcliffe EM. Group A meningococcal disease in England associated with the Haj. *J Infect* 1990;21:21-5.
- Riou JY, Caugant DA, Selander RK, Poolman JT, Guibourdenche M, Collatz E. Characterization of *Neisseria meningitidis* serogroup A strains from an outbreak in France by serotype, serosubtype, multilocus enzyme genotype and outer membrane protein pattern. *Eur J Clin Microbiol Infect Dis* 1991;10:405-9.
- Maiden MCJ, Malorny B, Achtman M. A global gene pool in the neisseriae. *Mol Microbiol* 1996;21:1297-8.
- Linz B, Schenker M, Zhu P, Achtman M. Frequent interspecific genetic exchange between commensal neisseriae and *Neisseria meningitidis*. *Mol Microbiol* 2000;36:1049-58.
- Morelli G, Malorny B, Müller K, Seiler A, Wang J, del Valle J, et al. Clonal descent and microevolution of *Neisseria meningitidis* during 30 years of epidemic spread. *Mol Microbiol* 1997;25:1047-64.
- Bolshakov KN. Epidemiological and geographical aspects of meningococcal infection [dissertation]. Moscow: Moscow Univ.; 1973.
- Kostyukova NN, Mironova TK, Leschinskaya EV. Microbiological characteristics of meningococcus infection in Moscow during a periodic rise in morbidity (1968-1971). *Zh Mikrobiol Epidemiol Immunobiol* 1974;5:29-32.
- Demina AA, Martinov JV. An analysis of the epidemic cycle of meningococcal infection in Russia (1969-1993). *Zh Mikrobiol Epidemiol Immunobiol* 1996;4:57-61.
- Sarkari J, Pandit N, Moxon ER, Achtman M. Variable expression of the Opc outer membrane protein in *Neisseria meningitidis* is caused by size variation of a promoter containing poly-cytidine. *Mol Microbiol* 1994;13:207-17.
- Zhu P, Morelli G, Achtman M. The *opcA* and *opcB* regions in *Neisseria*: genes, pseudogenes, deletions, insertion elements and DNA islands. *Mol Microbiol* 1999;33:635-50.
- Zhu P, van der Ende A, Falush D, Brieske N, Morelli G, Linz B, et al. Fit genotypes and escape variants of subgroup III *Neisseria meningitidis* during three pandemics of epidemic meningitis. *Proc Natl Acad Sci U S A*. In press 2001.
- Kwara A, Adegbola RA, Corrah PT, Weber M, Achtman M, Morelli G, et al. Meningitis caused by a serogroup W135 clone of the ET-37 complex of *Neisseria meningitidis* in West Africa. *Trop Med Int Health* 1998;3:742-6.
- Vogel U, Morelli G, Zurth K, Claus H, Kriener E, Achtman M, et al. Necessity of molecular techniques to distinguish between *Neisseria meningitidis* isolated from meningococcal disease and from their healthy contacts. *J Clin Microbiol* 1998;36:2465-70.
- Van Embden JDA, Cave MD, Crawford JT, Dale JW, Eisenach KD, Gicquel B, et al. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendation for a standardized methodology. *J Clin Microbiol* 1993;31:406-9.
- Keim P, Kalif A, Schupp J, Hill K, Travis SE, Richmond K, et al. Molecular evolution and diversity in *Bacillus anthracis* as detected by amplified fragment length polymorphism markers. *J Bacteriol* 1997;179:818-24.
- Keim P, Klevytska AM, Price LB, Schupp JM, Zinser G, Smith KL, et al. Molecular diversity in *Bacillus anthracis*. *J Appl Microbiol* 1999;87:215-7.
- Vieusseux M. Mémoire sur le maladie qui a régné à Genève au printemps de 1805. *J Med Chirurg Pharm* 1806;11:163-82.
- Swartley JS, Marfin AA, Edupuganti S, Liu LJ, Cieslak P, Perkins B, et al. Capsule switching of *Neisseria meningitidis*. *Proc Natl Acad Sci U S A* 1997;94:271-6.
- Parkhill J, Achtman M, James KD, Bentley SD, Churcher C, Klee SR, et al. Complete DNA sequence of a serogroup A strain of *Neisseria meningitidis* Z2491. *Nature* 2000;404:502-6.
- Scholten RJPM, Bijlmer HA, Poolman JT, Kuipers B, Caugant DA, van Alphen L, et al. Meningococcal disease in the Netherlands, 1958-1990: a steady increase in the incidence since 1982 partially caused by new serotypes and subtypes of *Neisseria meningitidis*. *J Infect Dis* 1993;16:237-46.
- Caugant DA, Høiby EA, Magnus P, Scheel O, Hoel T, Bjune G, et al. Asymptomatic carriage of *Neisseria meningitidis* in a randomly sampled population. *J Clin Microbiol* 1994;32:323-30.